

Development of the Urine Sugar Analysis Paper (USAP) method as a rapid tool for seed viability testing in okra (*Abelmoschus esculentus* L.)

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Abstract

Direct seed quality testing of okra through germination may take up to 21 days. Therefore, a rapid, simple, and inexpensive indirect biochemical method was developed. This study evaluated, for the first time in okra, the Urine Sugar Analysis Paper (USAP) as a rapid, simple, and inexpensive tool for assessing seed quality by measuring cell membrane leakage. The first experiment was to determine the effectiveness of the accelerated aging method at 40 °C compared to 45 °C and to use the aged lots of red okra with various levels of viability for testing using the USAP. Protein leakage, glucose, specific gravity, and pH of seed leachates were measured using USAP and compared with conventional vigor tests and spectrophotometric analyses in the second experiment. Accelerated aging at 45 °C effectively generated high, medium, and low viability seed lots. Incubation method at 35 °C for 18 h provided the clearest separation among viability levels. Under this condition, protein leakage detected by USAP was strongly positively correlated with spectrophotometric protein content and electrical conductivity, and strongly negatively correlated with germination percentage and speed of germination. These relationships indicate that greater protein leakage reflects lower physiological quality. This USAP method offers a rapid and inexpensive biochemical test that can reliably distinguish okra seed lots by viability and vigor, and represents a practical complement to standard germination tests.

Keywords: cell membrane leakage; glucose; protein; specific gravity; temperature

Introduction

Okra (*Abelmoschus esculentus*) is a horticultural crop widely consumed as a vegetable and used in diabetic medicine because of its bioactive compounds (Dantas *et al.*, 2021). All parts of okra contain nutrients such as vitamins, carbohydrates, flavonoids, protein, calcium, and other nutrients (Chaemsawang *et al.*, 2019). Okra seeds are rich in fat and protein, containing about 22.62% fat, 28.21% protein, and 17.28% lignin, which

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contribute to a rapid loss of viability during storage (Acikgoz *et al.*, 2016). High fat content accelerates oxidative processes that damage cellular structures and enzymes, thereby reducing seed viability and vigor (Bewley and Black, 2013). Ensuring a continuous supply of okra seeds with high physiological quality is therefore essential for stable crop production.

Seed testing is required to evaluate germination capacity and to ensure the availability of seed lots with high physiological quality suitable for cultivation (Widajati *et al.*, 2013). Direct seed testing through germination provides reliable estimates of viability and vigor but may take several days to weeks, thereby slowing seed production and marketing activities. Indirect seed testing focuses on changes in metabolic activity or cell membrane integrity during imbibition (Sadjad, 1994). Accelerated aging, conducted under high temperature and relative humidity, is widely used to generate differences in seed quality and to predict storability (ISTA, 2018). In okra, accelerated aging of Ahwaz exotype seeds at 40 °C reduced germination percentage from 96% to 49% after 192 h because of increased cell membrane leakage that interferes with seed metabolic processes (Parmoon *et al.*, 2019). Accelerated aging of okra cultivar Sabz Pari decreased the germination rate from 90% to 10% after 78 h at a seed moisture content of 12%. Glucose is leached from seeds and amylase activity is reduced during aging (Bakhtavar *et al.*, 2023). These studies indicate that membrane integrity and solute leakage are key physiological traits associated with the deterioration of okra seeds under stressful storage conditions.

Urine Sugar Analysis Paper (USAP) is developed for glucose detection in diabetic patients (Storey *et al.*, 2018). USAP strip is considered applicable for seed quality evaluation through the detection of membrane disintegration and leakage of solutes such as glucose and protein. USAP was able to detect glucose leakage after soaking cereal seeds for 6 h and barley seeds for 40 h (Takayanagi and Murakami, 1969). More recently, USAP has been used to distinguish seed with high and low viability using protein leakage and specific gravity of seed leachates as diagnostic parameter (Rukundo *et al.*, 2022; Mubarak *et al.*, 2025). However, although USAP has been tested in several crop species, no validated protocol is currently available for okra seeds. The optimal combinations of incubation time and temperature that allow USAP to clearly separate okra seed lots with contrasting viability, as well as the relationships between USAP parameters and conventional vigor tests under accelerated aging, have not yet been established. In addition, the validity of protein leakage measurements obtained with USAP needs to be confirmed by comparison with spectrophotometric protein assays, which are widely used for quantifying proteins in biological samples (Pokhrel *et al.*, 2020). Therefore, this study aimed to produce red okra seed lots with high, medium, and low viability through accelerated aging and determine the optimal incubation method for USAP to detect cell membrane leakage in these seed lots.

Materials and Methods

Seed Material and Preparation

The seeds used in this study were red okra obtained from the Indonesian Centre for Biotechnology and Biodiversity. Since 2016, the seeds have been multiplied by a grower in Bulugunung Village, Magetan Regency, East Java, Indonesia, at an altitude of 1,000 m above sea level. The okra seeds were harvested on 20 January 2024. The seeds were stored in airtight aluminium packaging at 20±2 °C in March 2025. The initial seed germination was 86%, and the seed moisture content was 11.91%. The type of USAP strip used was the URS-14 T, having 14 test parameters. The parameters assessed on USAP were protein, pH, and specific gravity.

Experiment 1: Accelerated aging test of okra seeds

Experimental design and treatment: The first experiment was arranged using a nested design, with temperature as the main factor and accelerated aging period nested within temperature. Accelerated aging was conducted at two temperatures, 40 °C and 45 °C. Accelerated aging period were set at 0 (control), 48 h, 96 h,

144 h, and 192 h. Each treatment was replicated four times, with 50 seeds per replicate. Temperatures of 40 °C and 45 °C and aging periods up to 192 h were selected based on previous studies on okra, which reported that these conditions effectively generate seed lots with contrasting viability while avoiding complete loss of germination. Seeds were cleaned and surface sterilized with 0.5% sodium hypochlorite for 1 min, then rinsed with distilled water and dried. Each box contained twenty grams of seeds laid out in a single layer to ensure consistent moisture uptake from the humid environment, after which the lid was placed on the boxes. These boxes were then placed on the oven with 2 cm of space between them to ensure even temperature distribution. After 192 h of aging, the boxes were removed from the oven. The seed moisture content of seeds after aging was measured using a low-constant-temperature oven method (ISTA, 2018).

Germination assay: Germination was evaluated using the between-paper (BP) method with four replications of 50 seeds per treatment. Seeds were incubated at 25 ± 2 °C for 21 days. Germination counts were recorded at 4 and 21 days after sowing, and only normal seedlings were considered for the calculation of germination percentage (El Balla *et al.*, 2011). The criteria for normal seedlings and the counting schedule followed ISTA (2018).

Germination parameters observed are:

Seed moisture content (SM)

Seed moisture content on a fresh weight basis was determined by the low-constant-temperature oven method. Seed sample (4.5-5 g) was dried at 103 ± 2 °C for 17 hours, and dry weight was recorded (ISTA, 2018). $(SM) = [(FW-DW)/FW] \times 100\%$. FW is fresh weight, and DW is dry weight.

Germination percentage (GP)

Germination percentage is the percentage of total normal seedlings at the first and final count (4 and 21) days after germination (DAG) (ISTA, 2018). $(GP) = [(\sum NS I + \sum NS II) / \text{total seed sown}] \times 100\%$. NS I is a normal seedling on the first count, while NS II is one on the final count.

Speed of germination (SG)

The speed of germination was determined by counting normal seedlings at 24-hour intervals from the day after germination until the last day of observation (Sadjad, 1994). Speed of Germination $(SG) = \sum_0^t (\% \cdot 24h^{-1})$.

Electrical conductivity (EC)

Seed samples were drawn, of which 50 seeds were counted, and their weight was recorded. These 50 seeds were soaked in a beaker glass having 150 mL of distilled water. Electrical conductivity of the distilled water should be less than $5 \mu S cm^{-1}$. After soaking the seeds, the beakers were covered with aluminium foil and then placed at 20 ± 2 °C for 24 h. The electrical conductivity of the solution was measured by using an electrical conductivity meter. The following equation was used to calculate EC in $\mu S cm^{-1} g^{-1}$ (ISTA, 2018; Amiruddin, 2024).

$$EC = \frac{\text{Conductivity reading} - \text{Background/blank reading}}{\text{Seed mass (g)}}$$

Seed structure (SS)

Seed structure was examined using a light microscope. Seeds without aging and seeds aged at 45 °C for 96 h and 192 h were transversely sectioned through the embryo region with a sharp cutter blade and immediately observed without staining at $2\times$ magnification under bright field illumination. For each viability class, the condition of the embryo, the compactness of the storage tissues, and the presence of cracks and cavities in the reserves were recorded as criteria for tissue degradation. Images were captured by positioning a smartphone camera over the microscope eyepiece and keeping the same magnification and illumination settings for all samples.

Experimental 2: Optimal incubation method for determining cell membrane leakage using USAP paper

The second experiment focused on the optimal incubation temperature and duration to detect cell membrane leakage in okra seeds using USAP. This experiment used okra seeds without aging, and those aged at 45 °C for 96 h and 192 h were used to represent high, medium, and low viability. The second experiment was arranged in a nested design, with incubation method as the main factor while seed viability was the nested factor. The treatments consisted of incubation at 35 °C for 12, 18, and 24 h; 25 °C for 12, 18, and 24h; and 18 °C for 12, 18, and 24 h. For each treatment, 50 seeds were cut into four pieces and placed in a glass jar containing 25 ml of distilled water. Seeds were cut to increase the exposed surface area and to promote rapid and uniform leaching of cellular solutes. Preliminary observations (data not shown) using intact okra seeds under the same incubation conditions indicated that solute leakage could not be detected within the incubation period, presumably because the very hard seed coat restricted water uptake and solute release. Therefore, seed pieces were used in all treatments. This procedure increases the absolute amount of leakage compared with whole seeds, but all viability classes were treated in the same way, so relative differences in membrane integrity among seed lots could still be compared. The seeds were incubated according to the treatment, filtered, and the soaking water was transferred to a 10 ml test tube. USAP strips were dipped into the soaking water for 3 min, wiped with tissue, and left for 1 min to allow color development. The color change of the strip was compared with the table on the URS-14T packaging to determine protein leakage, as well as changes in specific gravity and pH of okra seeds. Protein and glucose were analyzed by spectrophotometry on the USAP incubation method, which distinguished seed viability.

USAP parameters observed are:

Color change of the USAP strip (CC)

USAP strips were immersed in the seed soaking water, and the color changes were compared with the reference scale on the USAP URS-14T packaging. Color intensities were associated with protein, specific gravity, and pH of the soaking solution. The parameters were defined as follows: protein (0–0.3 g L⁻¹ = viable, 0.3–3.0 g L⁻¹ = less viable, 3.0–20 g L⁻¹ = dead); specific gravity (1.000–1.010 = viable, 1.015–1.020 = less viable, 1.025–1.030 = dead); pH (5.0–6.5 = viable, 7.0–7.5 = less viable, 8.0–8.5 = dead (Shabirah, 2022)).

Seed biochemical attributes (SB)

The protein content of seed leachate was analyzed using the Bradford method (Bradford, 1976) with Bovine Serum Albumin (BSA) as the standard. The glucose content of seed leachate was analyzed using the dinitrosalicylic acid (DNS) method (Miller, 1959).

Statistical analysis

The data was analyzed by analysis of variance (ANOVA) using SAS On Demand for Academics. If the results show a significant ($P < 0.05$), the means were subjected to Duncan's Multiple Range Test (DMRT) to determine the difference between means. Pearson correlation was applied for optimal results with physiological parameters and protein leakage from USAP with spectrophotometry.

Results and Discussion*Okra seed lots produced by accelerated aging*

Temperature and accelerated aging period clearly modified the physiological quality of okra seeds. At both 40 °C and 45 °C, seed moisture content increased from the initial level to around 20% at the longest aging period (Table 1). At 40 °C, moisture content rose up to 144 h and then changed only slightly, indicating that the seeds approached an equilibrium moisture content at which imbibition slowed down (Bakhtavar *et al.*, 2019). In contrast, at 45 °C moisture content continued to increase until 96 h, followed by a small decline at 192 h, suggesting membrane disruption and subsequent water loss after prolonged exposure to high

temperature. Similar increases in moisture content during accelerated aging have also been reported for chickpea and maize seeds (Araujo *et al.*, 2021; Sri and Nagarajappa, 2024).

Accelerated aging at 40 °C had no significant effect on reducing germination percentage and was less effective in decreasing seed viability and vigor because the seeds had not yet been exposed to a level of heat stress sufficient to induce physiological damage. The temperature of 40 °C is still below the critical threshold for reducing enzyme activity; therefore, deterioration occurs more slowly. This agrees with previous studies showing that 40 °C may require longer exposure to effectively reduce seed viability, and that its discriminating power depends on the species and seed lot used (Sultan *et al.*, 2020; Favoretto *et al.*, 2024). Therefore, 40 °C was less efficient for rapidly separating okra seed lots based on vigor.

Accelerated aging at 45 °C caused a progressive decrease in seed viability and vigor with increasing exposure time, indicating that high temperature effectively intensifies seed deterioration. Under these conditions, enhanced respiratory and oxidative reactions promote membrane disruption and other cellular damage, which is expressed as reduced germination, reduced germination speed, and increased electrolyte leakage. Similar responses were reported by Parmoon *et al.* (2019), who observed that germination of okra cultivars Ahwaz and Isfahan declined from above 85% to less than 50% after 192 h of aging, accompanied by decreases in catalase, peroxidase, and ascorbate peroxidase activities due to protein degradation, enzyme inactivation, and loss of membrane integrity. Barreto and Garcia (2017) also reported that 12 d of accelerated aging in macaw palm seeds enhanced malondialdehyde production, confirming the association between membrane leakage and lipid peroxidation during aging. These patterns show that accelerated aging generates seed lots with contrasting physiological quality, providing an appropriate vigor gradient for evaluating the sensitivity of the USAP method based on membrane leakage.

Table 1. Effect of accelerated aging temperature and period on moisture content, germination percentage, speed of germination, and electrical conductivity of okra seeds

Accelerated aging temperature	Accelerated aging period (hour)				
	0	48	96	144	192
Moisture content (%)					
40 °C	11.5 ± 0.05 d	17.0 ± 0.04 c	20.7 ± 0.12 a	19.9 ± 0.13 b	20.8 ± 0.41 a
45 °C	11.1 ± 0.06 c	18.2 ± 0.21 b	20.0 ± 0.88 a	18.6 ± 0.21 b	18.5 ± 0.02 b
Germination percentage (%)					
40 °C	84.0 ± 3.65 ab	88.0 ± 2.83 ab	90.5 ± 5.74 a	82.0 ± 4.89 ab	80.0 ± 8.64 b
45 °C	87.5 ± 5.26 a	78.5 ± 4.12 b	60.0 ± 6.73 c	56.5 ± 7.72 c	46.0 ± 1.63 d
Speed of germination (% 24h ⁻¹)					
40 °C	21.4 ± 2.21 b	22.5 ± 1.56 ab	26.0 ± 2.21 a	22.5 ± 2.70 b	19.2 ± 2.81 b
45 °C	20.0 ± 1.34 a	17.4 ± 0.97 b	15.4 ± 0.63 c	12.2 ± 0.66 d	10.1 ± 0.97 e
Electrical conductivity (μS cm ⁻¹ g ⁻¹)					
40 °C	40.4 ± 0.36 a	43.4 ± 0.64 ab	45.4 ± 0.58 bc	47.3 ± 1.27 c	53.8 ± 1.44 d
45 °C	45.8 ± 1.32 a	61.4 ± 2.63 b	66.6 ± 3.85 b	76.2 ± 3.99 c	78.2 ± 0.67 c

Means ± standard deviation; within each temperature, means in the same row followed by the same letter are not significantly different according to DMRT test, $p < 0.05$.

Seed structure of okra at different viability levels

Seed structure was examined to identify morphological and anatomical changes following accelerated aging. Observations were conducted on seeds without aging, aging at 45 °C for 96 h, and aging at 45 °C for 192 h, representing high, medium, and low viability, respectively. High viability seeds show an intact embryo and cotyledon, with cells remaining compact. Medium viability seeds show modest structural alterations, reflected in less compact cellular organization. Meanwhile, low viability seeds show severe structural deterioration marked by embryo degradation (Figure 1A-C).

These structural changes are consistent with increased metabolite leakage during prolonged aging. Yao *et al.* (2024) reported that accelerated aging of oat (*Avena sativa*) seeds for 96 h resulted in embryonic cells with reduced compactness, separation from the plasma membrane, and disorganized cellular structure. Low viability seeds were characterized by high protein leakage, attributed to loss of testa integrity during imbibition after aging. Similarly, Mandizvo and Odindo (2019) observed that the seeds of bambara groundnut landrace G340A, which have a relatively thin testa, exhibited elevated electrical conductivity and reduced viability after 120 h of aging.

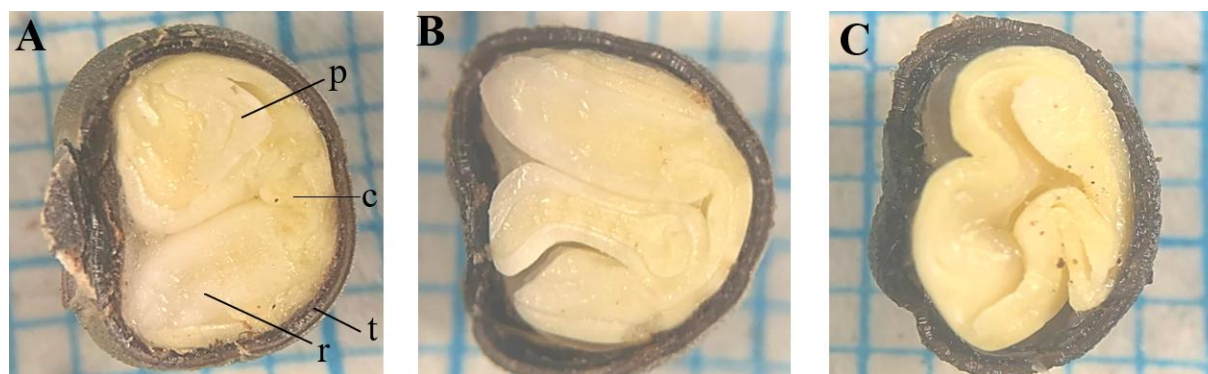


Figure 1. Structure of okra seeds with (A) no aging; (B) aging at 45 °C for 96 h; (C) aging at 45 °C for 192 h. Anatomical markers are indicated in (A); r: radicle; t: testa; c: cotyledon; p: plumule

Optimal incubation method for USAP testing

Okra seed lots with high, medium, and low viability obtained no aging, aging at 45 °C for 96 h and 192 h were incubated under different incubation method to optimize the USAP test. Protein leakage increased with incubation temperature and duration, and this increase was consistently greater in low viability seeds, indicating more extensive disruption of membrane integrity. Under incubation at 35 °C for 18–24 h and at 25 °C for 18 h, USAP readings clearly separated the three viability classes, whereas incubation at 18 °C for 18 h produced only small differences in leakage among seed lots. Low temperatures slow imbibition and solute diffusion so that deteriorated seeds do not leak sufficient protein to be distinguished from medium viability seeds. In contrast, moderately warm conditions promote leakage in low viability seeds while maintaining relatively low leakage in high viability seeds, thereby maximizing the discriminatory power of USAP. In addition, high viability seeds could be differentiated from medium and low viability seeds through solution specific gravity at 25 °C (18 h) and pH at 18 °C (24 h), confirming that multiple USAP parameters can be used to classify seed lots (Table 2).

Low seed viability is closely associated with loss of membrane integrity and oxidative damage. Fernandez *et al.* (2018) reported that *Salix* spp. seeds stored for a longer period exhibited lower germination and reduced protein stability compared with more recently harvested seeds, together with higher concentrations of malondialdehyde, a marker of lipid peroxidation. Elevated malondialdehyde content indicates deterioration of membrane lipids in low viability seeds, allowing proteins and electrolytes to leak more readily into the surrounding medium. Once membrane integrity is compromised, reactive oxygen species (ROS) further attack membrane lipids and proteins, disrupting essential biochemical and physiological processes (Bewley and Black, 2013). These mechanisms explain why deteriorated okra seeds in the present study released substantially more protein than high viability seeds under the same incubation method.

The sensitivity of the USAP method is influenced by high temperature, which accelerates metabolic activity, leading to cell membrane damage and enhanced differentiation of protein leakage among seed lots. Choudhary *et al.* (2024) showed that heat-treated fenugreek seeds exposed to 40 °C for 12 h exhibited markedly higher protein leakage than those incubated at 30 °C, supporting the view that moderate heat stress accelerates

solute loss from deteriorated seeds. In USAP, the color intensity of the test strip reflects the total concentration of solutes released from the seed solution, including proteins, sugars, organic acids, and other metabolites. Seed solution specific gravity and pH provide complementary information on this solute load (Hoy and Gamble, 1985), so that higher leakage from low vigor seeds translates into higher specific gravity, lower pH, and stronger USAP signals. Santos *et al.* (2019) demonstrated that pH testing distinguished the viability of seven rice seed lots incubated at 25 °C for 60 min. Together, these physiological relationships explain the observed protein leakage patterns and highlight why carefully selected incubation methods are critical for USAP to function as a rapid and effective assay of seed vigor.

The USAP method has been widely applied as a rapid technique for assessing seed quality in several crops. Mubarak *et al.* (2025) reported that USAP sensitivity to glucose was relatively low in peanut seeds. However, this study also reveals an important limitation of the USAP approach. Glucose concentrations in okra seed leachates were low, and no color change was detected on the glucose indicator pads, even for low vigor seed lots. This poor glucose sensitivity indicates that USAP should primarily be interpreted as a protein leakage test rather than a reliable indicator of soluble sugars, and it therefore complements rather than replaces other biochemical assays.

Table 2. Effect of incubation method and viability class on protein leakage, specific gravity, and pH detected using USAP strips

Incubation method	Viability class	Protein leakage (g L ⁻¹)	Specific gravity (-)	pH
35 °C 12 h	High	0.20 ± 0.09 b	1.021 ± 0.008	5.50 ± 0.41
	Medium	0.48 ± 0.29 ab	1.021 ± 0.007	5.50 ± 0.41
	Low	0.77 ± 0.40 a	1.024 ± 0.006	5.63 ± 0.48
35 °C 18 h	High	0.25 ± 0.09 c	1.020 ± 0.003 b	5.75 ± 0.29
	Medium	1.00 ± 0.00 b	1.023 ± 0.003 ab	5.63 ± 0.48
	Low	2.33 ± 0.58 a	1.028 ± 0.003 a	5.38 ± 0.48
35 °C 24 h	High	0.53 ± 0.20 c	1.019 ± 0.006	6.00 ± 0.41
	Medium	1.67 ± 0.58 b	1.025 ± 0.004	5.63 ± 0.48
	Low	2.68 ± 0.58 a	1.026 ± 0.003	5.63 ± 0.48
25 °C 12 h	High	0.10 ± 0.09 b	1.014 ± 0.003	6.13 ± 0.48
	Medium	0.15 ± 0.00 ab	1.011 ± 0.004	6.13 ± 0.25
	Low	0.37 ± 0.24 a	1.016 ± 0.001	6.00 ± 0.71
25 °C 18 h	High	0.05 ± 0.09 c	1.011 ± 0.002 b	6.13 ± 0.25
	Medium	0.25 ± 0.09 b	1.017 ± 0.001 a	6.00 ± 0.41
	Low	0.77 ± 0.20 a	1.021 ± 0.004 a	5.63 ± 0.25
25 °C 24 h	High	0.15 ± 0.15 b	1.014 ± 0.004	6.13 ± 0.25
	Medium	0.48 ± 0.29 ab	1.018 ± 0.007	5.75 ± 0.29
	Low	0.77 ± 0.40 a	1.021 ± 0.004	5.75 ± 0.29
18 °C 12 h	High	0.05 ± 0.09	1.010 ± 0.004	6.31 ± 0.24
	Medium	0.05 ± 0.09	1.012 ± 0.003	6.38 ± 0.25
	Low	0.20 ± 0.08	1.008 ± 0.002	6.38 ± 0.75
18 °C 18 h	High	0.05 ± 0.08 b	1.015 ± 0.005	6.25 ± 0.50 ab
	Medium	0.15 ± 0.15 b	1.010 ± 0.004	6.62 ± 0.63 a
	Low	0.30 ± 0.00 a	1.016 ± 0.004	5.75 ± 0.29 b
18 °C 24 h	High	0.10 ± 0.08	1.012 ± 0.002	5.62 ± 0.25 b
	Medium	0.32 ± 0.29	1.013 ± 0.001	6.00 ± 0.00 a
	Low	0.25 ± 0.08	1.014 ± 0.003	6.00 ± 0.00 a

Means ± standard deviation; within in incubation method, means in the same column followed by the same letter are not significantly different according to DMRT test, $p < 0.05$. Protein leakage values are presented in the original scale; data were square root transformed before ANOVA. Parameters without letters are not significantly different. High, medium, and low viability seed lots had germination percentages of 87.5%, 60.0%, and 46.0%, respectively

Three USAP incubation methods that effectively distinguished high, medium, and low seed viability were selected for spectrophotometric analysis of protein and glucose content in the seed leachates. In addition, the treatment at 18 °C for 18 h, which differentiated only high viability from medium and low, was included to represent the three incubation temperatures of, 35, 25, and 18 °C. This assay further substantiates the finding that specific incubation method can optimize the ability of USAP to discriminate among different viability levels of okra seeds.

Spectrophotometric measurements confirmed that protein leakage increased from high to low viability seed lots after incubation at 35 °C for 18 h (Table 3). This pattern reflects greater membrane deterioration in aged seeds, allowing storage proteins to diffuse into the soaking solution. Similar relationships between loss of membrane integrity, enhanced solute efflux, and reduced seed performance have been reported in other species. In *Brassica napus*, Yin *et al.* (2025) found that seeds subjected to a controlled deterioration test showed markedly higher ion leakage, lower germination, and reduced superoxide dismutase activity than non-deteriorated seeds. The decline in antioxidant activity indicates an accumulation of reactive oxygen species, which further damages membranes and promotes the release of intracellular ions and proteins.

Glucose content analysis at 35 °C for 24 h showed some separation among viability classes, whereas incubation at 25 °C and 18 °C for 18 h could only distinguish low viability seeds from high and medium viability seeds. The overall range of glucose leakage was narrow approximately 19–49 mg 100 g⁻¹, indicating that this parameter has limited power to discriminate among viability levels compared with protein leakage. The low sensitivity of glucose in this context is likely related to its minor functional role in seed metabolism and its very low abundance in okra seeds. These findings are in line with Kamaei *et al.* (2024), who reported only a small increase in glucose leakage after accelerated aging of *Allium cepa* seeds. Our results indicate that glucose leakage using USAP is not suitable for assessing okra seed viability, protein leakage provides a more sensitive and consistent indicator of membrane deterioration.

Table 3. Effect of viability class and incubation method on protein and glucose leakage from okra seeds measured by spectrophotometry

Viability class	Incubation method			
	35 °C 24 h	35 °C 18 h	25 °C 18 h	18 °C 18 h
Protein (mg 100 g ⁻¹)				
High	375.50 ± 0.47 b	344.28 ± 7.51 c	344.17 ± 13.41 b	316.72 ± 6.55 a
Medium	412.67 ± 9.19 a	380.67 ± 10.87 b	361.89 ± 5.29 a	316.61 ± 4.51 b
Low	415.66 ± 3.77 a	401.61 ± 2.46 a	339.83 ± 0.76 b	339.11 ± 6.55 b
Glucose (mg 100 g ⁻¹)				
High	29.52 ± 1.68 c	21.13 ± 0.11 b	19.99 ± 0.88 b	19.88 ± 0.57 b
Medium	38.34 ± 1.39 b	22.75 ± 1.36 b	22.89 ± 0.81 b	22.88 ± 0.81 b
Low	49.27 ± 0.70 a	43.85 ± 8.10 a	31.06 ± 3.19 a	34.81 ± 3.18 a

Means ± standard deviation; for each incubation method, means in the same column followed by the same letter are not significantly different according to DMRT test, $p < 0.05$. High, medium, and low viability seed lots had germination percentages of 87.5%, 60.0%, and 46.0%, respectively

Correlation of protein content measured using USAP with seed viability and vigor

Pearson correlation and simple linear regression were performed to determine the association between USAP results and seed viability and vigor of okra. The X variables consisted of incubation method, while the Y variables were seed viability and vigor. Out of the nine combinations, only 35 °C for 24 h, 35 °C for 18 h, and 25 °C for 18 h resulted in distinct separation of high, medium, and low viability seed lots according to USAP protein content. In the other incubation method, the differentiation of viability categories was not distinctly expressed. Therefore, these incubation methods were not subjected to the correlation analysis. Other variables, such as specific gravity and pH were not included in the correlation analysis because their responses were not

demonstrably sensitive to distinguish seed physiological quality and therefore contributed no further relevant information.

Table 4. Correlation between protein leakage detected by USAP and seed viability and vigor parameters of okra seeds under different incubation method

Incubation method	Viability and vigor	$\rho(r)$	Linear regression equation	R^2
35 °C for 24 h	Moisture content	0.523	$y = 6.7002x + 7.963$	0.274
35 °C for 24 h	Germination percentage	-0.835**	$y = -59.552x + 127.52$	0.697
35 °C for 24 h	Speed of Germination	-0.928**	$y = -13.282x + 28.307$	0.755
35 °C for 24 h	Electrical conductivity	0.894**	$y = 44.675x + 6.0115$	0.801
35 °C for 18 h	Moisture content	0.441	$y = 5.339x + 10.658$	0.194
35 °C for 18 h	Germination percentage	-0.873**	$y = -58.889x + 117.67$	0.762
35 °C for 18 h	Speed of Germination	-0.928**	$y = -13.424x + 26.468$	0.862
35 °C for 18 h	Electrical conductivity	0.957**	$y = 45.213x + 12.122$	0.916
25 °C for 18 h	Moisture content	0.320	$y = 8.2889x + 9.707$	0.103
25 °C for 18 h	Germination percentage	-0.797**	$y = -114.83x + 149.45$	0.635
25 °C for 18 h	Speed of Germination	-0.872**	$y = -26.94x + 34.409$	0.761
25 °C for 18 h	Electrical conductivity	0.918**	$y = 92.68x + 16.391$	0.844

r = Pearson correlation coefficient; R^2 = coefficient of determination for the linear regression between protein leakage (g L^{-1}) and each viability or vigor parameter. ** indicates a significant correlation at $p < 0.01$

The correlation results indicate that higher color intensity on the USAP strips reflects greater protein leakage from the seeds. This leakage is strongly associated with the deterioration of physiological quality in okra seeds. A similar pattern was reported by Souto *et al.* (2022), who found that electrical conductivity in *Libidibia ferrea* seeds was negatively correlated with 1000 seed weight, seedling height, seedling dry weight, and root length. Loss of membrane integrity leads to the leakage of essential metabolites, thereby reducing the seed's capacity to support germination. Consequently, protein leakage detected by the USAP method is closely associated with a reduced potential of seeds to develop into healthy and vigorous seedlings.

The highest correlation coefficients and coefficients of determination were obtained at 35 °C for 18 h, where protein leakage was strongly and negatively correlated with germination percentage and speed of germination, and strongly and positively correlated with electrical conductivity. These results indicate that USAP-detected protein leakage can reliably predict standard physiological parameters and support the selection of 35 °C for 18 h as the most informative incubation method for rapid okra seed quality screening. The need to optimize incubation conditions so that leakage tests show the strongest association with seed quality is consistent with previous electrical conductivity studies, in which temperature, water volume, and incubation duration were adjusted to maximize the test quality relationship. For example, in wheat cultivars Sakha 95, Sakha 94, and Misr 3, the EC test was optimized using 5 g of seeds incubated in 25 mL of distilled water at 25 °C for 24 h (Kishk and Elbatrawy, 2023), while for sunflower seeds of the IL01 variety, the best EC conditions were 50 seeds in 38 mL of distilled water at 25 °C for 23 h (Szemruch *et al.*, 2019).

Correlation between protein leakage measured using USAP and spectrophotometry

Pearson correlation and simple linear regression were used to analyze the relationship between USAP and spectrophotometric measurements of protein leakage. The results show that incubation method at 35 °C for 18 h has a strong, positive, and significant correlation (Figure 2). An increase in the color intensity of protein leakage on the USAP reflects elevated protein concentrations as measured by spectrophotometry. The correlation coefficient is 0.918 with a coefficient of determination R^2 is 0.843, indicating that 84% of the variability in protein measurement obtained through spectrophotometry can be explained by the USAP method.

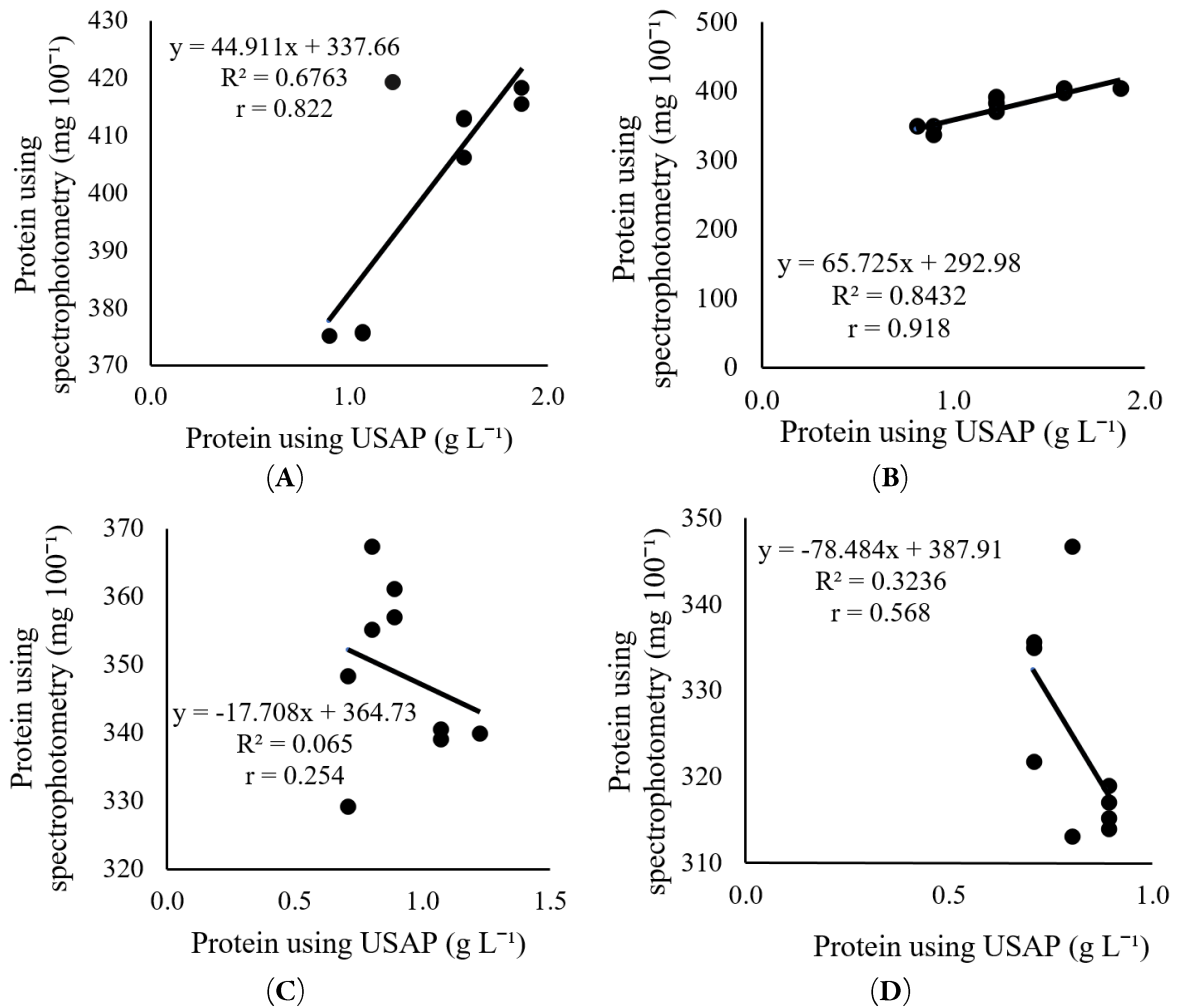


Figure 2. Relationship between protein leakage measured by USAP and protein content measured by spectrophotometry under different incubation methods; (A): 35 °C for 24 h; (B): 35 °C for 18 h; (C): 25 °C for 18 h; (D): 18 °C for 18 h. Points represent replicate values per viability level. Regression lines, equations, r , and R^2 are shown

Biochemical components of seeds including lipid, glucose, and protein can be used as important indicators of seed quality. In wheat and maize, seed protein content has been reported to correlate strongly and significantly with the dry weight of normal seedlings, with correlation coefficients of 0.897 and 0.894 respectively. This result demonstrates a strong association between protein reserves and seed germination capacity. Proteins provide amino acids for enzyme synthesis and seedling structure development during the early stages of germination. Their degradation during imbibition supplies energy to support metabolic processes. Consequently, low protein content reduces the nutrient supply required for germination and decreases seed viability and vigor (Wen *et al.*, 2017).

The USAP method provides a rapid, inexpensive, and feasible method for detecting protein leakage in okra seeds, particularly for early-stage screening prior to more complex laboratory analyses. The visible color change clearly reflects the extent of protein leakage. This method has the potential to be applied by agricultural practitioners in the field or in basic laboratories for preliminary seed quality assessment, thus reducing the time and cost required for testing.

Color change of USAP (Urine Sugar Analysis Paper)

The color pattern on USAP was evaluated by observing changes on the reagent paper. Protein leakage was detected by a color transition from yellow to greenish blue. A yellow paper indicates normal conditions, reflecting intact seed cell membranes and high viability. The transition towards bluish green was observed when proteins and organic compounds leached from seeds into the paper as a result of the loss of membrane integrity. The intensity of the green coloration increases with declining seed viability, higher incubation temperature, and prolonged incubation time. This indicates an increase in protein leakage associated with cellular physiological impairment. The color developed on USAP reflects metabolic activity during incubation, functioning as a visual indicator to distinguish the physiological condition of seeds with high, medium, and low viability. Low viability seeds produce a dark green color, aligned with the USAP color standard range of 0.3-10 (Figure 3). The most intense green coloration is observed under the treatments of 35 °C for 18 h and 25 °C for 18 h.

The color change in USAP is determined by an acid-based chemical mechanism involving tetra bromophenol blue (TBPB). Under normal conditions, the indicator appears yellow and shifts to green or blue as pH increases due to the release of charged compounds from the seeds. Seed proteins contain amino acid groups with positive charges (NH_3^+). Electrostatic interactions between the anion of USAP and the positively charged amino acid in proteins alter electro distribution, resulting in a visible color change (Suzuki, 2007). Other studies have shown that tetra bromophenol blue is a stable and sensitive indicator paper that can be used for protein detection in biological samples. This paper is considered one of the most effective tools for protein detection compared with other methods (Pokhrel *et al.*, 2020).

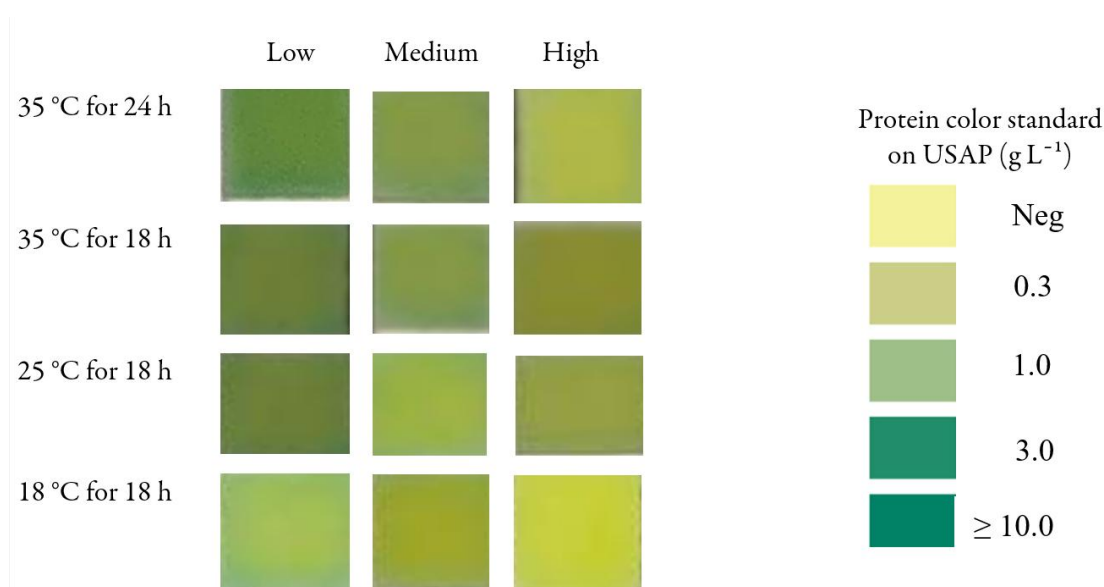


Figure 3. Protein indicator color changes on USAP strips for soaking solutions of okra seeds under different incubation methods and viability levels. Rows represent incubation methods (35 °C for 24 h, 35 °C for 18 h, 25 °C for 18 h, and 18 °C for 18 h), and columns represent low, medium, and high viability seed lots. The panel on the right shows the USAP protein color standard

The performance of the optimized USAP incubation method at 35 °C for 18 h can also be evaluated in relation to the standard electrical conductivity (EC) test. In our study, seed lots that exhibited higher EC values, indicating greater membrane leakage of electrolytes, also showed higher protein leakage as detected by USAP, with strong and significant positive correlations between protein leakage and EC and negative correlations with viability and vigor parameters (Table 4). These relationships suggest that the USAP method and the EC test diagnose the same underlying process of membrane deterioration in aged seeds. However, USAP strips offer

several practical advantages, including low cost, wide availability, and rapid visual scoring without the requirement for a conductivity meter. Thus, the optimized USAP protocol at 35 °C for 18 h can serve as a simple alternative or complementary tool to the EC test for routine screening of okra seed lots, particularly in laboratories with limited equipment.

Conclusions

Accelerated aging at 45 °C for 0 h, 96 h, and 192 h successfully produced okra seed lots with high, medium, and low viability, respectively. USAP assays at 35 °C for 24 h and 18 h and at 25 °C for 18 h effectively discriminated these viability levels based on protein leakage, whereas changes in specific gravity and pH were less consistent. Among the incubation methods, 35 °C for 18 h was the most effective condition, showing strong positive correlations between protein leakage detected by USAP, spectrophotometric measurements, and seed physiological quality. In contrast, glucose leakage exhibited only a narrow variation and did not clearly separate viability classes, indicating that protein leakage is a more reliable indicator of okra seed viability than glucose. USAP strips are low-cost, widely available, and require only simple incubation facilities. The optimized protocol at 35 °C for 18 h has good potential for rapid seed quality screening in resource-limited laboratories.

Authors' Contributions

Conceptualization: TRAS, AQ, and MRS; Laboratory work and data curation: TRAS, Technical Supervision: AQ, MRS; Data analysis and interpretation: TRAS, AQ, MRS; Manuscript writing: TRAS; Manuscript review: AQ, MRS. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article

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